APPLICATION FOR UNITED STATES LETTERS PATENT
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(Case No. 02-1106-A)

Title: **Detection Of *Salmonella* Cells By Fluorescence Polarization Inhibition Assays**

Inventors: Michael E. Jolley
34469 North Circle Drive
Round Lake, Illinois 60073

(Citizen of the United Kingdom)

Mohammad Sarwar Nasir
1012 Highgate Lane
Grayslake, Illinois 60030

(Citizen of the United States)

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/419,020, filed on October 16, 2002, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the field of diagnostic assays. More particularly, this invention relates to an inhibition assay that uses fluorescence polarization for the detection of *Salmonella* cells.

2. Description of Related Art

Fluorescence polarization is a useful technology for the detection of the binding of small molecules to larger ones. The underlying principle of the technology is that fluorescence polarization can serve as a measure of the rate of rotation of fluorescent molecules and, hence, their size. In particular, small molecules have a fast rotation rate and therefore tend to have a low fluorescence polarization, whereas large molecules rotate more slowly and therefore tend to have a higher fluorescence polarization. The principles and applications of fluorescence polarization have been described in a recent review. *See* M.S. Nasir and M.E. Jolley, *Combinatorial Chemistry and High Throughput Screening*, 1999, *2,* 177-190.

Fluorescence polarization assays have been developed for the detection of serum antibodies to *Brucella abortus* (*See* K. Nielsen, et al., *J. Immunol. Methods*, 1996, *195,* 161-168), *Mycobacterium bovis* (*See* M. Lin, et al., *Clin. Diagnos. Lab. Immun.*, 1996, *3,*

438-443), equine infectious anemia virus (*See* S.B.Tencza, et al., *J. Clin. Microbiol.*, 2000, *38,* 1854-1859), and *Salmonella enteriditis* (*See* M.S. Nasir, et al., *Proceedings of the 104th Annual Meeting of the United States Animal Health Association*, 2000, 527-535).

However, in order to screen for *Salmonella* contamination in food products, it is desirable to detect *Salmonella* cells directly. Currently, this is often done by first culturing a sample, such as on an agar plate. The culture is then tested for *Salmonella* by exposure to antiserum in an agglutination assay. Such agglutination assays, however, are not quantitative (indeed, they are potentially subjective) and often suffer from low sensitivity. Accordingly, there is a need for objective and sensitive assays specific for *Salmonella* cells that are rapid and easy to perform.

SUMMARY OF THE INVENTION

In a first principal aspect, the present invention provides a method for detecting *Salmonella* antigens in a sample. In accordance with the method, the sample is combined with a tracer and an anti-*Salmonella* antibody to form an assay mixture. The tracer comprises a fluorophore conjugated to an oligosaccharide from a *Salmonella* cell wall lipopolysaccharide. The tracer is able to bind to the anti-*Salmonella* antibody to produce a detectable change in fluorescence polarization. The fluorescence polarization of the assay mixture is measure to obtain a measured fluorescence polarization value. The measured fluorescence polarization value is related to the concentration of *Salmonella* antigens in the sample.

In a second principal aspect, the present invention provides a method for testing for *Salmonella* contamination. In accordance with the method, a sample containing *Salmonella* cells is obtained. The sample is cultured in a culture medium to provide a cultured sample. The cultured sample is then autoclaved to provide an autoclaved sample. The autoclaved sample is combined with anti-*Salmonella* antibody to provide a first mixture. The fluorescence polarization of the first mixture is measured to obtain a first fluorescence polarization value. The first mixture is combined with a tracer to provide a second mixture. The tracer comprises a fluorophore conjugated to an oligosaccharide from a *Salmonella* cell wall lipopolysaccharide. The tracer is able to bind to the anti-*Salmonella* antibody to produce a detectable change in fluorescence polarization. The second mixture is incubated for a predetermined period of time. The fluorescence polarization of the mixture is measured to obtain a second fluorescence polarization value. The first fluorescence polarization value is subtracted from the second fluorescence polarization value to obtain a corrected fluorescence polarization value. The corrected fluorescence polarization value is related to the level of *Salmonella* contamination in the sample.

In a third principal aspect, the present provides an assay kit for testing for *Salmonella* contamination in a sample. The assay kit comprises an anti-*Salmonella* antibody and a tracer, each in an amount suitable for at least fluorescence polarization assay to test for *Salmonella* concentration in the sample, packaging, and instructions for using the anti-*Salmonella* antibody and tracer in the fluorescence polarization assay. The tracer comprises a fluorophore conjugated to an oligosaccharide from a *Salmonella* cell

wall lipopolysaccharide. The tracer is able to bind to the anti-*Salmonella* antibody to produce a detectable change in fluorescence polarization.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plot showing the measured fluorescence polarization of the SE.1, SE.5, ST.01, and ST.1 tracers after varying amounts of the AB1 antiserum were added.

Figure 2 is a plot showing the measured fluorescence polarization of the SE.1 tracer after varying amounts of the AB2 antiserum were added and of the ST.01 tracer after varying amounts of the AB3 antiserum were added.

Figure 3 is a plot showing the measured fluorescence polarization for samples containing varying levels of *Salmonella enteriditis* cells (using the AB1 antiserum and the SE.1 and SE.5 tracers) and for samples containing varying levels of *Salmonella typhimurium* cells (using the AB1 antiserum and the ST.01 and ST.1 tracers).

Figure 4 is a plot showing the measured fluorescence polarization for samples containing varying levels of *Salmonella enteriditis* cells (using the AB2 antiserum and the SE.1 and SE.5 tracers) and for samples containing varying levels of *Salmonella typhimurium* cells (using the AB3 antiserum and the ST.01 and ST.1 tracers).

Figure 5 is a plot showing the measured fluorescence polarization of *Salmonella newport* and *Salmonella montevideo* cultured on agar plates.

Figure 6 is a plot showing the measured fluorescence polarization of *Salmonella newport* and *Salmonella montevideo* cultured on agar plates.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a homogeneous fluorescence polarization inhibition assay for detection of *Salmonella* cells in samples, such as samples from food products or animal feces. The sample may be cultured in a culture medium, such as a broth or an agar plate, before the assay is performed. The assay makes use of a tracer comprising a fluorophore conjugated to an oligosaccharide from a *Salmonella* cell wall lipopolysaccharide. A sample suspected of containing *Salmonella* cells is added to an anti-*Salmonella* antibody to form a mixture, and a blank fluorescence polarization measurement is taken. The tracer is then added to the mixture. After incubation, the fluorescence polarization of the mixture is measured and the blank reading is subtracted. The level of *Salmonella* contamination in the sample may be determined from the fluorescence polarization measured in this way.

This fluorescence polarization approach has been found to be very sensitive, specific, rapid, quantitative, inexpensive, and robust. Moreover, in exemplary embodiments, the tracers used in this inhibition assay to detect *Salmonella* cells are the same as may be used to detect serum antibodies to *Salmonella*. Accordingly, *Salmonella* detection kits may be used both for screening for *Salmonella* infection in live animals, by detecting serum antibodies, and for testing for the presence *Salmonella* cells in food products and animal feces.

EXAMPLE 1
(FPIAs for broth-cultured *Salmonella entiriditis* and *Salmonella typhimurium*)

Fluorescence polarization inhibition assays (FPIAs) for the detection of *Salmonella enteriditis* (SE) and *Salmonella typhimurium* (ST) cells have been developed

using three antisera and four fluorescently-labeled preparations (tracers) of the O-polysaccharides (OPSs) from SE (SE.1 and SE.5) and ST (ST.01 and ST.1). A bovine anti-ST antiserum (AB1) recognized all tracers. A specific rabbit anti-*Salmonella* Group D1 antiserum (AB2) recognized the SE tracers but did not react with the ST tracers. Conversely, a specific rabbit anti-*Salmonella* Group B antiserum (AB3) recognized the ST tracers but not the SE tracers. None of the tracers recognized rabbit specific anti-*E. coli* O157 or anti-*Salmonella* Group E antisera (all O-antigens). Chicken specific anti-*Salmonella pullorum* (SP) antisera reacted strongly to these tracers.

Cells were grown in culture overnight and autoclaved (15 minutes at 121° C). 20 µl of autoclaved sample was added to one ml of an appropriately diluted antiserum in buffer and a fluorescence blank obtained (Sentry-FP™ instrument; Diachemix Corp.). To this mixture was added 10 µl of appropriately diluted tracer. The reaction mixture was incubated at room temperature for 4 minutes, and the blank-subtracted fluorescence polarization (FP) of the tracer determined. A sample having a fluorescence polarization less than 10 mP of that of medium alone (5 standard deviations: SDs) was considered positive.

None of the assays cross-reacted with *Salmonella montevideo* (SM; Group C1) or *Escherichia coli* (EC; two strains). All assays had a dynamic range of less than 10^5 to approximately 10^7 CFUs. Assays that used the AB2 and AB3 antisera were totally specific to SE and to ST, respectively. When the AB1 antiserum was used, the ST tracers were approximately 10% cross-reactive with SE, and the SE tracers were approximately 0.5% cross-reactive with ST.

1. Materials and methods

The lipopolysaccharides (LPSs) from SE and ST were obtained from Sigma Chemical Co., St. Louis, MO (catalogue numbers L 6011 and L 6511 respectively). Fluorescein isothiocyanate, isomer I (FITC-I) and media were also obtained from Sigma (catalogue number F 7250). EC strains were obtained from Becton Dickinson Microbiology Systems, Cockeysville, MD. They were catalogue numbers 4337015 and 4337071 (ATCC numbers 25922 and 35218 respectively; EC#1 and EC#2). SE, ST, and SM strains used in the FPIAs were field isolates from chickens.

Bovine anti-ST (Poly Serum®) was provided by Grand Laboratories, Larchwood, IA. Rabbit specific anti-*Salmonella* D1 (O:1,9,12), B (O:1,4,5,12) and E (O:1,3,10,15,19,34) were obtained from Difco Laboratories, Detroit, MI (cat. nos. 229511, 22481 and 228191 respectively). Rabbit specific anti-*E.coli* O157 was purchased from BBL, Sparks, MD (cat. no. 0263950). Chicken specific anti-SP (high and low titer and negative) were obtained from NVSL, Ames, IA.

The preparation of the tracer, i.e., fluorescently-labeled O-polysaccharides (OPSs) is described in M.S. Nasir et al., *Proceedings of the 104th Annual Meeting of the United States Animal Health Association*, 2000, 527-535, which is incorporated herein by reference. Briefly, the OPSs were obtained by hydrolysis of the corresponding LPSs by hydrolysis in 1% acetic acid in a boiling water bath and purification by passage through a polymyxin B-agarose column. Fluorescein was attached by alkalization and reaction with FITC-I, purification by Sephadex G25 gel chromatography and A25 anion exchange chromatography. Tracers SE.1 and SE.5 were eluted with 0.1 M sodium phosphate buffer, pH 7.5, and 0.5 M sodium chloride, in the same buffer, respectively. Their yields

were approximately equal. Tracers ST.01 and ST.1 were eluted with 0.01 M and 0.1 M sodium phosphate, pH 7.5, respectively. Again, the yields were approximately equal.

SE, ST, and SM cells were grown in tryptic soy broth overnight at 37° C. EC cells were similarly grown in EC medium. Cultures were killed by autoclaving at 121° C for 15 minutes before assaying. 20 µl of the autoclaved culture was added to 1 ml of diluted antiserum in PBSA (0.01 M sodium phosphate, pH 7.5, containing 9 g/l sodium chloride and 0.1% sodium azide). A fluorescence blank was taken (Sentry-FP™, Diachemix Corp., Grayslake, IL) and 10 µl of tracer, diluted in PBSA-BGG (PBSA containing 100 µg/ml bovine gamma globulin) such that a 1:100 dilution gave approximately 1 nM fluorescein equivalents, was then added. The blank-subtracted fluorescence polarization of the tracer was then determined after four minutes.

2. Results

The reactions of antisera with tracers SE.1, SE.5, ST.01, and ST.1 are summarized below in Table 1. Unless otherwise stated, the antisera were employed at a dilution of 1:50. It was found that AB2 reacted only with SE tracers and AB3 reacted only with ST tracers, as indicated by the delta mP columns in Table 1. Surprisingly, however, AB1, which was raised against ST, reacted significantly better with the SE tracers than with the ST tracers. Chicken anti-SP antisera reacted well with both SE.5 and ST.1 (SE.1 and ST.01 were not tested), showing the immunological similarity of SE and SP. Rabbit anti-Group E and anti-EC O157 showed no significant reaction with either of the SE or ST tracers that were tested.

	SE.1 (mP)	Delta mP	SE.5 (mP)	Delta mP	ST.01 (mP)	Delta mP	ST.1 (mP)	Delta mP
Buffer	93		97		96		93	
AB1 (1/100)	245	153	246	149	180	84	186	93
Buffer	88		96		99		90	
AB2 (1/50)	170	82	181	85	96	-3	88	-2
AB3 (1/50)	88	0	95	-2	141	42	141	51
O157 (1/50)	92	4	100	4	99	0	90	0
Buffer	NT		105		NT		98	
Anti-SP -	NT		114	9	NT		98	0
Anti-SP +	NT		177	72	NT		144	46
Anti-SP ++	NT		285	180	NT		240	142
Anti Gp E	NT		108	3	NT		94	-4

Table 1. Assessment of the binding of the tracers with various antisera (NT = not tested).

Figure 1 shows the fluorescence polarization (in mP) as the tracers ST.01, ST.1, SE.1 and SE.5 were titrated with AB1. Figure 2 shows the fluorescence polarization as SE.1 was titrated with AB2 and ST.01 was titrated with AB3. Surprisingly, SE.1 and SE.5 were equivalent with AB1, as were ST.01 and ST.1. Previous studies have found that the low salt tracers are significantly inferior to the high salt tracers in the detection of chicken antibodies. For this reason, only SE.1 and ST.01 were used for the AB2 and AB3 titrations.

Table 2 below shows the results of the SE.1 and SE.5 tracers, with antisera AB2 and AB1, in FPIAs for ST, SM, EC (two strains), and various levels of SE. Table 3 below shows the results for the ST.01 and ST.1 tracers, with antisera AB3 and AB1, in FPIAs for SE, SM, EC (two strains), and various levels of ST.

			AB2 Rabbit anti-GP D1 (1:50)			AB1 Bovine anti-ST (1:300)	
Sample	CFUs (x 10^{-5})		SE.1 (mP)	SE.5 (mP)		SE.1 (mP)	SE.5 (mP)
ST	200		176	190		156	161
SM	200		174	184		172	178
EC #1	>>200		ND	ND		175	ND
EC #2	>>200		ND	ND		162	175
SE	200		110	119		101	107
SE	100		116	130		103	110
SE	50		126	140		103	115
SE	25		138	150		111	122
SE	12		149	161		118	126
SE	6		160	168		127	137
SE	3		165	172		141	146
SE	1.5		167	175		152	158
SE	0.8		170	179		166	167
SE	0.4		172	180		172	172
Medium	0		177	185		173	179

Table 2. Results of FPIAs using tracers SE.1 and SE.5 and antisera AB2 and AB1 (ND = not determined).

			AB3 Rabbit anti-GP B (1:25)			AB1 Bovine anti-ST (1:100)	
Sample	CFUs (x 10^{-5})		ST.01 (mP)	ST.1 (mP)		ST.01 (mP)	ST.1 (mP)
SE	200		166	171		122	128
SM	200		166	172		159	172
EC #1	>>200		ND	ND		160	175
EC #2	>>200		ND	ND		162	175
ST	200		120	111		107	103
ST	100		127	116		109	108
ST	50		133	125		113	114
ST	25		141	134		118	122
ST	12		147	143		127	132
ST	6		149	150		136	144
ST	3		156	158		147	154
ST	1.5		159	161		152	162
Medium	0		166	173		162	173

Table 3. Results of FPIAs using tracers ST.01 and ST.1 and antisera AB3 and AB1 (ND = not determined).

Figure 3 is a plot of the results of the FPIAs of SE, using antiserum AB1 and tracers SE.1 and SE.5, and of ST, using antiserum AB1 and tracers ST.01 and ST.1. Figure 4 is a plot of the results of the FPIAs of SE, using antiserum AB2 and tracers SE.1 and SE.5, and of ST, using antiserum AB3 and tracers ST.01 and ST.1. The standard deviations of the media in these assays were approximately 2mP (data not shown). Therefore, a mP of medium alone minus 10 mP was chosen as the cutoff (99.9% confidence level). The assays had sensitivities of 10^{-5} CFUs, or less, and dynamic ranges of approximately two orders of magnitude. The assays using the SE tracers and the AB1 antisterum had a cross-reactivity with ST of approximately 0.5%, and the assays using

the ST tracers and the AB1 antiserum had a cross-reactivity with SE of approximately 10%. The assays that used the AB2 and AB3 antisera were totally specific to SE and to ST, respectively. SM and EC (two strains) exhibited no cross-reactivity in any of the assays.

3. Conclusions

It has been found that *Salmonella enteriditis* (SE) and *Salmonella typhimurium* (ST) cells may be detected in fluorescence polarization inhibition assays (FPIAs) employing fluorescently-labeled O-polysaccharides (OPSs) from the corresponding bacterial lipopolysaccharides (LPSs) as tracers and appropriate antisera. The FPIAs are very rapid, sensitive, and simple to perform. They may also be quantitative and made to be field portable. Furthermore, the reagents used in the FPIAs are very stable and low cost.

EXAMPLE 2
(FPIAs for detecting *Salmonella* cells in colonies cultured on agar plates)

The FPIA approach has also been shown to be able to detect *Salmonella* cells in colonies cultured on agar plates. The overall approach is as follows. Samples suspected of containing *Salmonella* cells are cultured on agar plates until visible colonies develop. A colony is then removed from the plate, suspended in 1 ml of water and boiled for five minutes. 100 μl is then added to 1 ml of a solution containing antibodies specific to the *Salmonella* species being tested (e.g., an appropriate antiserum diluted in buffer), and the mixture is mixed well. A blank fluorescence polarization measurement is taken of the mixture and then 10 μl of tracer is added. The resulting mixture is then incubated for 2

minutes, and its fluorescence polarization is measured, subtracting the blank measurement.

Table 4 below shows the results for FPIA assays conducted on *Salmonella newport* (SN1 and SN2) and *Salmonella montevideo* (SM1 and SM2) cultured on "ONOZ" and "Brilliant Green" agar plates. These assays were performed using tracers C1 and C2 prepared from the OPSs of *Salmonella montevideo* and *Salmonella newport*, respectively, in a manner similar to that described above with respect to *Salmonella enteriditis* and *Salmonella typhimurium*, and the corresponding antisera. Figure 5 graphically illustrates the results of Table 4. Table 5 below shows similar FPIA results, except that the *Salmonella montevideo* and *Salmonella newport* were cultured on "XLT4" and on "SS" agar plates. Figure 6 graphically illustrates the results of Table 5.

	ONOZ Agar				Brilliant Green Agar			
	C2 tracer (mP)	Delta mP	C1 tracer (mP)	Delta mP	C2 tracer (mP)	Delta mP	C1 tracer (mP)	Delta mP
PBSA	181		180		186		186	
SN1	142	-39	180	0	139	-47	184	-2
SN2	126	-55	180	0	138	-48	182	-4
SM1	181	0	113	-67	187	1	133	-43
SM2	183	2	121	-59	182	-4	105	-81

Table 4. FPIA results for *Salmonella newport* and *Salmonella montevideo* cultured on agar plates.

	XLT4 Agar				SS Agar			
	C2 tracer (mP)	Delta mP	C1 tracer (mP)	Delta mP	C2 tracer (mP)	Delta mP	C1 tracer (mP)	Delta mP
PBSA	184		190		184		190	
SN1	140	-44	187	-3	141	-43	184	-6
SM1	177	-7	136	-54	187	1	133	-43
PBSA	178		184		178		184	
SN2	115	-63	177	-7	112	-66	178	-6
SM2	172	-6	121	-63	172	-6	98	-86

Table 5. FPIA results for *Salmonella newport* and *Salmonella montevideo* cultured on agar plates.

FPIA assays were also performed on *Salmonella typhi* (STy21a) samples cultured on "Brilliant Green" agar plates. These FPIAs assays used the *Salmonella enteriditis* tracer SE.5 and the *Salmonella typhimurium* tracer ST.1 described above, along with corresponding antisera. The results are summarized in Table 6 below.

	SE.5 tracer (mP)	Delta mP	ST.1 tracer (mP)	Delta mP
PBSA	176		168	
STy21a-2-1	145	-31	169	1
STy21a-2-2	130	-46	169	1

Table 6. FPIA results for *Salmonella typhi* cultured on agar.

These results show that the FPIA approach may be used to detect many different *Salmonella* species, whether they are cultured in broth or on agar plates.

EXAMPLE 3
(Assay kit)

The materials used to perform the assay of the present invention may be made available in kit form. The kit preferably includes tracer and anti-*Salmonella* antibody in an amount suitable for at least one assay, along with suitable packaging and instructions for using the tracer and antibody to test for *Salmonella* contamination in a sample. The sample could be from a food product or from animal feces, for example. The sample could also be cultured in a culture medium, such as an agar plate or a broth, before the assay is performed on it.

The tracer and antibody may be provided in solution, as a liquid dispersion, or as a substantially dry powder (e.g., in lyophilized form). The suitable packaging can be any solid matrix or material, such as glass, plastic, paper, foil, and the like, capable of separately holding within fixed limits the buffer, tracer, and antibody. For example the tracer and antibody may be provided as solutions in separate labeled bottles or vials made of glass or plastic.The tracer comprises a fluorophore, such as fluorescein isothiocyanate, isomer I, conjugated to an oligosaccharide from a *Salmonella* cell wall lipopolysaccharide. Other fluorophores may be used, provided the resulting tracer is able to bind with the anti-*Salmonella* antibody to produce a detectable change in fluorescence polarization.

The foregoing description of the invention is presented for purposes of illustration and description, and is not intended, nor should be construed, to be exhaustive or to limit the invention to the precise forms disclosed. The description was selected to best explain the principles of the invention and practical application of these principles to enable others skilled in the art to best utilize the invention in various embodiments and with

various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention not be limited by the specification, but defined by the claims.